

ERYTHROMYCIN

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IDENTITY

Chemical names International Union Pure and Applied Chemistry (IUPAC) name:
 6-(4-dimethylamino-3-hydroxy-6-methyl-tetrahydropyran-2-yl)oxy-14-ethyl-7,12,13-trihydroxy-4-(5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl)oxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione

C.A.S. number 114-07-8

Synonyms and abbreviations

Mixture of macrolide antibiotics, the main component (erythromycin A) being (3R, 4S, 5S, 6R, 7R, 9R, 11R, 12R, 13S, 14R)-4-[(2,6-dideoxy-3-C-methyl-3-O-methyl- α -L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[3,4,6-trideoxy-3dimethylamino- β -D-xylo-hexopyranosyl)-oxy]oxacyclotetradecane-2,10-dione

Structural formula See next page.

Molecular formula C₃₇H₆₇NO₁₃

Molecular weight **Erythromycin A:** Mz = 734; **Erythromycin B:** Mz = 718;
Erythromycin C: Mz = 720

OTHER INFORMATION ON IDENTITY AND PROPERTIES

Appearance White or slightly yellow powder or colorless or slightly yellow crystals

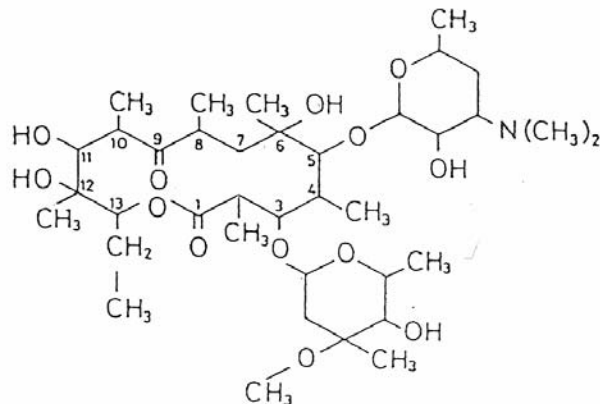
Degree of purity Erythromycin B \leq 5.0%
 Erythromycin C \leq 5.0%
 Any impurity \leq 3.0%
 Sum of impurities \leq 7.0%

Qualitative composition of impurities

N-demethyl-erythromycin A, anhydroerythromycin A, erythromycin A enol ether, pseudoerythromycin A enol ether, erythromycin E, erythromycin F.

Description of physical properties

Slightly hygroscopic, slightly soluble in water but less soluble at higher temperatures, freely soluble in alcohol, soluble in methanol, sensitive to light.



Three erythromycins are produced during fermentation. Erythromycin A and B contain the same sugar moieties, desoxamine and cladinose (3-O-methylmycarose). They differ in position 12 of the aglycone, erytronolide, A having a hydroxyl group. Erythromycin C contains desoxamine and the same aglycone present in A, but differs by the presence of mycarose instead of cladinose (Merck Index). The principal product is erythromycin A with small proportions of B and C.

INTRODUCTION

Erythromycin was first reviewed by the Committee in 1968. No ADI was established but acceptable levels of residues were defined in milk (0-0.04 mg/ml), meat and eggs (0-0.3 mg/kg) (FAO/WHO, 1969).

Pharmacokinetic and metabolic studies in experimental animals, target animals and humans were evaluated, including original studies in calves and chicken and three new non radiolabelled residue depletion studies in chickens, two in laying hens and one in turkeys and the description and validation of the analytical methods used.

Conditions of use

General

Erythromycin, a macrolide antibiotic is effective *in vitro* against *Mycoplasma*, Gram positive *Cocci*, *Neisseria*, some strains of *Haemophilus*, *Corynebacterium*, *Listeria*, *Pasteurella multocida*, *Brucella*, and *Treponemes*. *Proteus*, *Pseudomonas* and *E.Coli* are relatively resistant to the drug. In veterinary medicine, this compound is used for the treatment of clinical and subclinical mastitis in lactating cows, for the treatment of infectious diseases due to erythromycin sensitive bacteria (cattle, sheep, swine, poultry) and for the treatment of chronic diseases due to mycoplasma in poultry (EMEA, 2000). In Europe, for broiler chickens and laying hens, the most often recommended dose (as erythromycin base) is 20 mg/kg/day. Uses and dose ranges are presented in Table 1.

Table 1: Formulation and dose ranges of erythromycin

Formulation	Country	Dosage
Erythromycin thiocyanate Oral powder 5 g per 100 g	Belgium France Germany	20 mg / kg / day for 5 days
	Netherlands	20 mg / kg / day for 5 to 7 days
	International zone*	5 g of erythromycin in 100 liter of drinking water for 3 to 5 days
Erythromycin thiocyanate Oral powder 5.1 g per 100 g	Greece	Curative treatment: 2-4 g of product / liter of drinking water Preventive treatment: 1-2 g of product / liter of drinking water
Erythromycin thiocyanate Oral powder 10 g per 100 g	France	20 mg / kg / day for 3 days
	Algeria	10 g of erythromycin / 100 liter of drinking water for 3 to 6 days
Erythromycin thiocyanate Oral powder 16.5 g per 100 g	Ireland and United Kingdom	25.5 mg / kg / day for 1 to 5 days
Erythromycin thiocyanate Oral powder 20 g per 100 g	France	20 mg / kg / day for 3 days
	Greece	50 mg / kg / day for 3 days
	Spain	130 mg erythromycin / liter of drinking water or 0.65 g erythromycin / liter of water For CRD: treatment for 5 days. For infectious coriza and sinusitis: treatment for 7 days
	International zone*	20 g of erythromycin in 200 liter (prevention) or 100 liter (treatment) of drinking water for 3 to 5 days
	International zone*	5 g of erythromycin per liter of drinking water for 1 to 5 days
Erythromycin Phosphate Oral powder 29.6 g per 100 g	Ireland and United Kingdom	25.5 mg / kg / day for 1 to 5 days

* Countries other than the European Union and United States

PHARMACOKINETIC AND METABOLIC STUDIES

Absorption

Erythromycin base is destroyed by gastric acid, except if administered with a protective enteric coating. Acidic media degrades erythromycin rapidly to form derivatives with little antimicrobial activity. Erythromycin stearate is more stable, however *in vitro* studies have demonstrated that erythromycin stearate dissolves in gastric acid, retains only 2% antibiotic activity and is rapidly destroyed (DiSanto and Chodos, 1981; Periti et al., 1989; Martindale, 1989). The major site of absorption in rat, dogs and humans is the small intestine. Erythromycin is only slightly absorbed from the stomach. In man, absorption occurs mainly in the duodenum (Anderson et al., 1959; Huber, 1977).

Humans

In humans, erythromycin is rather slowly absorbed after oral administration. Peak serum concentrations occur 1 to 6.3 hours after dosing and vary from 0.1 to 4.8 µg/ml, depending on the formulation, and the coating of erythromycin administered. The oral absorption is less than 50% and

erythromycin is degraded by gastric acid. It is absorbed in the small intestine as erythromycin base (DiSanto and Chodos, 1981; Griffith and Black, 1970; Burrows, 1980).

The oral bioavailability of unprotected erythromycin base and salts is less than 50 % of the dose. Food reduces the absorption of erythromycin (Griffith and Black, 1970; Burrows, 1980). Wilson and Van Boxtel (1978) observed that erythromycin propionate and stearate were better absorbed before rather than after breakfast.

Laboratory animals

In laboratory animals, erythromycin is rather slowly absorbed after oral administration in laboratory animals (except rats). Oral administration of propionyl erythromycin (25 mg/kg) in rats did not produce high peak serum concentrations ($<0.1 \mu\text{m}/\text{ml}$). However, the maximum serum concentration was reached rapidly (1 hour after administration). At the end of six hours following oral administration, only a trace of antibiotic activity was found in rat serum (Anderson et al., 1959).

Calves

In calves, 2 hours after a single intramuscular treatment of 5 mg erythromycin/kg b.w., the mean highest concentration in plasma ($0.652 \mu\text{g}/\text{ml}$) was reached. Twelve hours after treatment, the concentration of erythromycin in serum was about $0.22 \mu\text{g}/\text{ml}$. After repeated intramuscular treatments of 5 mg erythromycin/kg bw/day for 5 days, no accumulation phenomenon was observed (Report PK 5251/E-00).

Chickens

In chickens, 30 minutes after the beginning of a repeated administration of erythromycin via drinking water at a dose of 25,000 IU/kg b.w./day for 3 days (approximately 27 mg/kg b.w.), the average serum levels ranged from 0.11 to $0.22 \mu\text{g}/\text{ml}$. After the last administration, serum levels declined to approximately $0.04 \mu\text{g}/\text{ml}$ (Report PK 8400/E-00).

Distribution

Plasma protein binding

In humans, erythromycin is highly bound to plasma proteins. The extent of protein binding is $>74\%$ *in vitro* and $>90\%$ *in vivo* (Wilson and Van Boxtel, 1978). Erythromycin undergoes a relatively low extent of binding to bovine serum proteins (37-43%) (Baggot and Gingerich, 1976).

Milk protein binding

Studies show that antibiotics are bound only to a minor extent to milk proteins. However, the unbound fraction of erythromycin may be decreased because it may be bound to milk casein. Erythromycin is $<25\%$ bound to dry udder secretion and to dry udder tissue homogenates (Ziv, 1980).

Serum levels

According to Wilson and Van Boxtel (1978), dose levels of 250 mg of erythromycin base or erythromycin stearate in adults produce similar peak serum concentrations ($0.4 \mu\text{g}/\text{ml}$) within 2-4 hours after oral administration. In cattle, after intramuscular administration of erythromycin in cattle, peak serum concentrations are maintained for several hours and then decline slowly. The 12-hour levels are about 25% of peak concentration (Burrows, 1980).

The elimination half-life ($T_{1/2}$) of erythromycin following intravenous injection of a single dose in different species is shown in Table 2.

Table 2: Elimination half-life of erythromycin following intravenous injection

Species	Dose	$T_{1/2}$	References
Cow	12.5 mg/kg	3.16 ± 0.44 hours	Baggot and Gingerich, 1976
Dog	-	1 hour	Burrows, 1980
Man	100 mg	1.02 ± 0.17 hours	Wilson and Van Boxtel, 1978

Tissue distribution

Animal studies indicate that erythromycin is well distributed in the body and tissue levels (e.g. liver, spleen, kidneys, and lungs) are generally higher than serum levels and persist longer (Wilson and Van Boxtel, 1978).

Humans

In humans, erythromycin is distributed to various tissues and fluids. About 10% of erythromycin is estimated to cross the placenta and fetal blood levels are no higher than 10% (usually closer to 2%) of those present in normal circulation. An estimated 0.1% of a daily dose appears in breast milk in pregnant women (Wilson and Van Boxtel, 1978).

Rats

In rats given 100 mg erythromycin base per kg bw orally, erythromycin is concentrated in the liver, sub maxillary glands, spleen, adrenals, lungs and kidneys two hours after administration. Large amounts are also found in the thymus, skin, muscle, reproductive organs and heart (Lee et al., 1953).

Twenty hours after an intravenous treatment of 10 mg erythromycin (N-methyl- ^{14}C -erythromycin, 8 μCi) to rats, about 37-43% of the administered dose is recovered in the intestinal tract plus feces, 27.2 - 36.1% in the urine, and 21-29% in the expired air. It is rapidly metabolized in the liver, mainly through a demethylation process, and excreted in the bile as des-N-methyl-erythromycin, the major metabolite, present only in the bile and in the intestinal contents of rats. The isotopic methyl group is eliminated in the expired air as CO_2 (Lee et al., 1953)

Cattle

After intravenous administration, erythromycin is widely distributed in cows. The apparent volume of distribution (V_d) is 0.8 liter/kg. Tissue concentrations are higher than serum concentrations and erythromycin concentrations in milk of lactating cows (the dose fraction recovered in milk is 3.8%. At 6 hours, the percent of the dose of erythromycin in the central and tissue compartments were 6 and 19%, respectively, with 75% of the dose eliminated (Wilson and Van Boxtel, 1978; Ziv, 1980a). Compared to adult cows, a larger apparent volume of distribution and a higher body clearance rate were determined in calves (Burrows, 1980).

In lactating cows, erythromycin was well distributed in the body and mean erythromycin concentrations in renal cortex, muscle and liver varied from 0.09 to 0.14 $\mu\text{g/g}$ tissue, 16 hours after a single intramammary application of 1200 mg erythromycin base. The highest concentration was observed in the liver (Nouws and Ziv, 1979).

Five hours after intramuscular administration of erythromycin anhydrate (8.3 mg/kg bw) in cows, renal, muscle and liver concentrations were 0.11 to 0.92 $\mu\text{g/g}$, with the highest values in the liver. At a

dose of 9 mg/kg, concentrations were less than 0.03 to 0.06 µg/g; 67 hours after intramuscular injection of erythromycin base, the renal cortex concentration was 0.1 µg/g following a dose of 17.5 mg/kg (Nouws and Ziv, 1979).

Metabolism

The metabolism of erythromycin has been studied in different animal species and in humans. (Lee et al, 1956a; Lee et al, 1956b; Wilson and Van Boxtel, 1978; Pineau et al., 1990; Tsubaki and Ichikawa, 1985). These studies show that erythromycin is rapidly metabolized in the liver, mainly through an N-demethylation process in both rats and dogs and in the liver microsomal system of rabbits. Collectively, these studies strongly suggest that the metabolism of erythromycin by N-demethylation occurs in all species tested. Des-N-methyl-erythromycin is the major metabolite and the only microbiologically active metabolite of erythromycin. However, the antimicrobial activity is presumably low and the only form of erythromycin known to be active *in vivo* is the free base. It is excreted in the bile and eliminated through the faeces. Only erythromycin was found in the liver and the absence of des-N-methyl-erythromycin indicates that it is excreted in the bile immediately after erythromycin demethylation. It is absorbed from the intestinal tract but the very minute amount of des-N-methyl-erythromycin available in the body may explain its absence from urine.

The hepatic cytochrome P-450 isozymes that catalyse erythromycin demethylation in rat are highly similar to the form of liver cytochrome P-450 present in rabbit, hamster, gerbil and mouse and this may also extend to humans since human liver contains a protein equivalent to the rat cytochrome P-450. Similarly, a high degree of similarity was found between the ovine cytochrome P-450 involved in N-demethylation of erythromycin and the form isolated in rabbits. This suggests that an equivalent form of these liver cytochrome P-450 isozymes, with similar catalytic activities, is present in the species tested. In cattle a form of cytochrome P-450 isozyme exhibiting a high catalytic activity for N-demethylation was found; this activity was not measured for erythromycin but for other substrates having a N-methyl group structure.

Excretion

Renal excretion

In humans, the portion of an erythromycin dose excreted in the urine varies from 0.02 to 20% and the elimination half-life may be prolonged in renal disease. However, except complete renal failure, renal impairment has only a minor impact on the pharmacokinetics of erythromycin (Wilson and Van Boxtel, 1978).

Urinary excretion of erythromycin accounts for approximately 10% of an administered oral or IM dose (Burrows, 1980). Twenty hours after administration of isotopic erythromycin in rats, 27 to 36% of the radioactivity was recovered in the urine (Lee et al., 1956a).

Faecal Excretion

In humans, 15% of an administered dose was excreted in the bile (Griffith and Black, 1970).

In rats, erythromycin and its metabolites are excreted mainly by way of bile, but in part, also by direct passage through the intestinal wall (Baggot and Gingerich, 1976). Two hours following intravenous injection of isotopic erythromycin, 15.1% of the dose was excreted in the bile (Lee et al, 1956b). Twenty hours following intravenous injection of isotopic erythromycin, 37-43% of the radioactivity is recovered in the intestinal tract plus faeces (Lee et al. 1956a). An enterohepatic recirculation may also contribute to the high concentrations of erythromycin in faecal samples (Kroboth et al., 1982).

Pharmacokinetic studies in calves and poultry

Calves

A 1988 study (Report PK 5251/E-00) was performed in calves to determine:

- The pharmacokinetics and bioavailability of erythrocin (erythromycin thiocyanate) injectable following single and multiple IM administrations
- Pulmonary levels after a single IM administration of erythromycin
- Residues of erythromycin in tissues after multiple IM administration of injectable erythrocin

Erythromycin concentrations were assayed by a microbiological method on agar medium using *Micrococcus Luteus* as the sensitive organism (LOD: 0.02 IU/ml in serum; 0.16IU/g in all tissues).

Erythromycin was administered as a single intravenous injection to five calves at a dose of 5 mg erythromycin activity/kg. The study demonstrated a large apparent volume of distribution (V_d area, or $V_d\beta$ 1.95 l/kg), a short mean residence time (MRT 2.36 h) and an efficient ability of the organism to remove the drug (Cl 0.77 l/kg/h). As a second experiment in the same assay, a single intramuscular injection of a dose of 5 mg erythromycin activity/kg was administered to seven calves. Good bioavailability was observed (F 95%). Compared to the intravenous route, the elimination half-life and volume of distribution was apparently increased. This could be related to a slow-rate of absorption from the injection site.

In another study, three calves received five consecutive intramuscular injections in the neck or gluteal muscle at a dose of 5 mg/kg at 24-h intervals. No accumulation was observed. The peak concentrations observed after each injection were similar to the C_{max} values obtained after a single injection.

In a third study, ten calves received a single intramuscular injection in the neck or into the gluteal muscle alternatively at a dose of 5 mg/kg erythromycin during five days. Drug levels in lungs were always higher than in serum. These data are consistent with the well-known higher tissue than serum concentrations of erythromycin.

In the fourth study, ten calves received a single intramuscular injection in the neck or into the gluteal muscle alternatively at a dose of 5 mg/kg erythromycin during five days. Liver, kidney, muscle non-injection sites, the last three injection sites and fat tissues were collected at different withdrawal times after the last injection. Five days after the last injection, the tissues were free of antibiotic residues except some injection sites. Seven days after the end of the treatment, all tissues, including injection sites were negative. One calf slaughtered ten days after the last injection confirmed these results.

Poultry

A study (Report PK 8400/E-00) was performed in poultry to determine:

- Pulmonary and blood levels of erythromycin after a drinking water medication for three consecutive days
- Residues of erythromycin in chicken tissues after administration by oral route in drinking water for three consecutive days
- Residues of erythromycin in whole hen eggs after a drinking water medication for seven consecutive days

In the first study, 168 adult broilers were given erythromycin thiocyanate in their drinking water for 3 consecutive days. The treated water was changed every day but the water consumption during treatment was not measured. Assuming that the water consumption was similar during the treatment period and before treatment, where water consumption was 235ml/chicken on average, the dose could be estimated to be 25 mg/kg/day. Blood and lung samples (2-10 birds/sampling time) were collected before the first administration and at:

- 30 min, 1h, 2h, 3h, 4h, 6h, 8h, 9h and 24h on day 1 and 2 of medication

- 30 min, 2h, 3h, 4h, 6h, 8h, 9h and 24h on day 3 of medication
- 3h, 4h, 6h, 8h, 9h and 12h after the end of medication

The microbiological method using *Micrococcus Luteus* as the sensitive organism was used to determine blood and lung levels (LOD: 0.02 IU/ml of serum; 0.2 IU/g tissue). Similar but low serum values were noted during the whole treatment period (0.03-0.2 IU/ml, on average), although large variations were observed between broilers, possibly due to differences in individual water consumption. Higher pulmonary (0.5-1 IU/g) than serum levels were observed during the three days treatment, with maximum levels occurring 4 - 12 hours after each changing of medicated water and an elimination time of eight hours. In samples collected 12 hours after the end of the medication, pulmonary levels were below the limit of detection.

In the second study, 15 adult broilers received one dose of erythromycin thiocyanate estimated to be 25 mg/kg/day by the oral route in their drinking water for 3 consecutive days with the treated water changed every 24 hours. At 3, 5, 7 and 10 days after the end of the treatment, the chickens were slaughtered and liver, muscle, fat and skin tissues were collected. The microbiological method noted above was used (LOD: 0.2 IU/g tissue). In all cases the amounts of erythromycin in chicken tissues were below the detection limits 3 days after the end of the treatment.

In the third study, 40 laying hens received the same treatment as the broilers in the previous experiment for seven consecutive days. Five eggs were collected at 3, 5 and 7 days during treatment. Erythromycin was determined in the whole egg with the microbiological method (LOD: 0.06 µg/ml whole egg). Concentration levels of the drug were below the detection limits six days after the end of the treatment.

RESIDUE DEPLETION STUDIES IN TARGET ANIMALS AND IN HENS' EGGS

No radiolabelled study was performed. The following new original residue depletion studies with unlabelled erythromycin were performed in poultry:

Table 3: Residues depletion studies with erythromycin in target animals and eggs

Species	Dose	Study number
Chicken	20 mg / kg / day for 3 days	MPK/5814/9812 MPK/Erythromycin/9957
	20 mg / kg / day for 8 days	MPK/5814/0301
	50 mg / kg / day for 3 days	MPK/210H1/0148
Laying hens	20 mg / kg / day for 3 days	MPK/5814/9908 MPK/Erythromycin/9961
	20 mg / kg / day for 7 days	MPK/5814/0417
Turkey	20 mg / kg / day for 3 days	MPK/5814/0225

The maximum recommended therapeutic dose is 20/mg/kg/day. The route of administration, the dose and the species are those intended for therapeutic use. These studies were performed in accordance with GLP and the European Community guidelines 87/18/EEC and 88/320/EEC, including all supplements published up to the day of the corresponding study start.

Residue Depletion Studies in Chicken

- Chickens treated for three consecutive days at the maximum recommended dose (MPK/5814/9812 and MPK/Erythromycin/9957)

A residue study was performed in order to assess the depletion of erythromycin and its metabolites (N-desmethyl erythromycin A) in edible tissues of broiler chickens after repeated administration. The test product was erythromycin thiocyanate 20% oral powder. Thirty six chickens (18 males and 18 females plus 6 extra birds to replace any that might become ill and not meeting the inclusion criteria were selected for the 7 day acclimatization period) were treated by oral administration of 20 mg/kg/day of erythromycin for 3 consecutive days. All birds belonged to the same strain, TR 551, and were healthy when they received the treatment. For the entire duration of the study, the experimental broiler chickens were in premises at temperature and hygrometry ranging from 16 - 20 °C and 46 - 76%, respectively. Animals were kept in individual cages and all animals were exposed to an alternating cycle of illumination (12 hours of light followed by 12 hours of darkness). Animals received an individual daily ration of maize, soya, sunflower and wheat supplemented with vegetables and had free access to water. They weighed 0.9±0.1 kg at the beginning of the treatment. Six animals per sampling time were slaughtered at 1 day, 2 days, 3 days, 4 days and 5 days after the end of treatment. Individual edible tissues collected from animals were: 100g of pectoral muscle, 100g of liver, 100g sample of kidneys and 100g of fat and skin in natural proportions.

A specific HPLC method coupled to a mass spectrometer detection system (LC/MS/MS) was used to determine erythromycin A, B and C and N-desmethyl-erythromycin-A in chicken tissues. Antimicrobial activity was measured by a microbiological plate assay. Erythromycin B and C were not detected in all samples, therefore only erythromycin A and N-desmethyl-erythromycin-A were reported. Mean concentrations of erythromycin A measured by both methods were below the LOD or the LOQ for all tissues at 1, 2, 3, 4 and 5 days after the end of the treatment (LOQ: 100 µg/g for all edible tissues and for both methods; LODs for the LC/MS/MS method were: 25 µg/kg for kidney, 30 µg/kg for liver, 3 µg/kg for muscle and 5 µg/kg for skin + fat; the LOD for the microbiological method was 50 µg /kg for all tissues). Mean concentrations of N-desmethyl-erythromycin A are presented in the following table.

Table 4: Mean concentrations of N- desmethyl-erythromycin A in edible tissues of broiler chickens receiving 20mg/kg/day for 3 days

Slaughter time after the end of treatment	Muscle (µg/kg)		Fat/skin (µg/kg)		Liver (µg/kg)		Kidney (µg/kg)	
	HPLC	Micro	HPLC	Micro	HPLC	Micro	HPLC	Micro
1 day	< LOD	< LOD	< LOQ	< LOD	< LOD	< LOD	< LOD	< LOD
2 days	< LOD	< LOD	< LOD	< LOD	282*	< LOD	< LOD	< LOD
3 days	< LOD	< LOD	< LOD	< LOD	163*	< LOD	< LOD	< LOD

Note: * one value

Results also show that only small concentrations of N-desmethyl-erythromycin A were measured by HPLC in liver up to 3 days in only two individual samples after the end of the treatment. For days 4 and 5, concentrations of the metabolite were under the LOD. Because the depletion rate of the residue was very rapid in all edible tissues, it was not possible to provide any correlation between both methods in broiler chickens.

Following the selection of Erythromycin A as the residue marker in edible tissues of broiler chickens, the following additional residue depletion studies were performed in the broiler chickens with different doses.

- Chickens treated for eight consecutive days at the maximum recommended dose (MPK/5814/0301)

Thirty six broiler chickens (18 males and 18 females plus 6 extra birds to replace any that might become ill and not meet the inclusion criteria) were selected for the acclimatization period (10 days) before the beginning of the treatment. The animals had an approximate age of 8 weeks at start of treatment and a mean weight of 1794g (1423-2154g). They were fed *ad libitum* with a pelleted concentrate ration and had free access to water. Chickens were treated by oral administration of 20% erythromycin A thiocyanate via drinking water at 20 mg/kg/day of erythromycin for 8 consecutive days. Six animals per sampling time were slaughtered at 12 hours, 1, 2, 3 and 4 days after the last administration. Individual edible tissues were collected from animals as follows: 400g sample of breast/leg muscle, entire liver, both kidneys and 200g sample of fat and skin in natural proportions.

The concentrations of residues in edible tissue were analyzed with the same LC/MS/MS validated method as the above reported study. Mean concentrations of erythromycin A were below the LOD or the LOQ for all tissues at 12 hours, 1, 2, 3, and 4 after the end of the treatment. The study demonstrated that, whatever the duration of administration, the 20 mg/kg b.w. dose of erythromycin, leads to concentrations of residues in edible tissues below the limit of quantification.

- Chickens treated for five consecutive days at two and one-half times the maximum recommended dose (MPK/210H1/0148)

A total of 36 broiler chickens (18 males and 18 females plus 6 extra birds to replace any might become ill and not meet the inclusion criteria) were selected for the acclimatization period (10 days) before the beginning of the treatment. The study was performed with a dose of 50 mg/kg body weight. The test product was a powder containing 5.5% erythromycin thiocyanate. The animals had an approximate age of 6 weeks at start of treatment and a weighed 800-1000g. They were fed *ad libitum* with a commercial feed and had free access to water. Chickens were treated by oral administration via drinking water for five consecutive days. Six animals per sampling time were slaughtered at 6h, 10h, 24h, 2, 5 and 7 days after the end of treatment. Individual edible tissues collected from animals were: 200g of muscle, entire liver, both kidneys and 40g of fat and skin in natural proportions. Erythromycin A was assayed with the same validated LC/MS/MS method as in the two previous studies. The results are presented in Table 5.

Table 5: Mean concentrations of Erythromycin A in edible tissues of chickens treated with 50mg/kg/day for 5 days

Sampling time after the end of treatment (hours)	Muscle (µg/kg)	Fat/skin (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)
6	133 ± 16	131 ± 35	3220 ± 2080	308 ± 170
10	< LOQ	< LOQ	1760 ± 2840	185 ± 79
24	< LOD	< LOQ	631 ± 393	< LOD

Note: < LOQ: below the quantification limit (100 µg/g for all edible tissues);

< LOD: below the detection limit (25 µg/kg for kidney, 30 µg/kg for liver, 3 µg/kg for muscle and 5 µg/kg for skin + fat).

Concentrations of erythromycin were measurable in all tissues at six hours after the end of the treatment but only in liver and kidney at ten hours after the end of the treatment (the concentrations in muscle and fat/skin were less than the LOQ). Residues at one day after the end of treatment were only found in liver; concentrations in other tissues were below the LOD or LOQ. At day 2 or further times post-treatment, concentrations in all tissues were below the LOQ or the LOD. The study demonstrates that the administration of doses higher than the recommended during consecutive days does not result in accumulation of residues.

Residue Depletion Studies in Laying Hens

- Laying hens treated for three consecutive days at the maximum recommended dose (MPK/5814/9908 and MPK/erythromycin/9961)

A residue study was performed in eggs from laying hens that received a repeated administration for three days. A total of 40 laying hens were selected for the acclimatization period (14 days) before the beginning of the treatment, 30 were selected for treatment according to individual egg production. From these, 25 laying hens were selected for sampling, keeping 5 animals to replace any that might become ill. All birds belonged to the same strain and were healthy when they received the treatment; no animal had received any treatment ten days before the beginning of the study. The laying hens were treated by oral administration of 20 mg/kg/day of erythromycin thiocyanate 20% oral powder. The laying hens weighed 2.1 ± 0.2 kg and were approximately 8 months at the beginning of the treatment. They were fed *ad libitum* with a commercial feed and had free access to water. All eggs produced were collected daily during the treatment and ten days after the end of the treatment. The production ranged from 19-26 eggs per day and 13-17 eggs per laying hen over the 16-day experimental period. Control samples were collected three days before the beginning of the treatment period. The total antimicrobial activity of residues was also determined with the microbiological plate assay. Test samples were a mixture of albumen and yolk of each egg at each time point. Only Erythromycin A and N-desmethyl-erythromycin-A were detected. Results are reported in the following table.

Table 6: Mean concentrations of erythromycin A and N-desmethyl-erythromycin A in eggs of laying hens treated with 20mg/kg/day for 3 days

Sampling time	Mean concentration erythromycin A ($\mu\text{g}/\text{kg}$)		Mean concentration N-desmethyl-erythromycin A ($\mu\text{g}/\text{kg}$)	Ratio HPLC / microbiological method for erythromycin A	Ratio HPLC / microbiological method for desmethyl-erythromycin A
	HPLC	Micro			
Beginning of treatment					
Day 0	109 \pm nc	194 \pm nc	< LOD	0.56	-
Day 1	78 \pm 12	158 \pm 76	< LOD	0.46	-
Day 2	83 \pm 24	198 \pm 76	< LOD	0.33	-
End of treatment					
1 day post treatment	57 \pm 6	221 \pm 80	97 \pm nc	0.23	-
2 day post treatment	71 \pm 6	207 \pm 59	120 \pm nc	0.25	0.76
3 day post treatment	54 \pm nc	118 \pm 21	69 \pm nc	-	-
4 day post treatment	< LOQ	< LOD	64 \pm nc	-	-
5 day post treatment	< LOQ	< LOD	< LOD	-	-
Mean				0.33	0.76

Note: <LOD: below the limit of detection (0.92 $\mu\text{g}/\text{kg}$ for the HPLC method and 50 $\mu\text{g}/\text{kg}$ for the microbiological method); <LOQ: below the limit of quantification (50 $\mu\text{g}/\text{kg}$ for the HPLC method and 100 $\mu\text{g}/\text{kg}$ for the microbiological method); nc: non calculated

Erythromycin A could only be quantified in 25% of eggs at day 1 and 12.5% of the eggs at day 2. Concentrations of erythromycin A after the end of the treatment measured by LC/MS/MS were near the LOQ of the method (50 $\mu\text{g}/\text{kg}$). N-desmethyl-erythromycin A had high concentrations but it has a very low antimicrobial activity. Both compounds were below the LOQ (50 $\mu\text{g}/\text{kg}$) six days after the end of treatment. Erythromycin A was identified as the marker residue for eggs.

- Laying hens treated for seven consecutive days at the maximum recommended dose (MPK/5814/0417)

Residues in eggs from laying hens were analyzed after repeated administration for seven days. The hens weighed 1.6 ± 0.16 kg and were approximately 23 weeks at the beginning of the treatment. Twenty laying hens were treated by oral administration of 20 mg/kg/day of erythromycin thiocyanate 20% oral powder. All birds belonged to the same strain and were healthy when they received the treatment after an acclimatization period of 21 days. The laying hens were fed *ad libitum* with wheat and a pelleted concentrated ration and had free access to water. Eggs were collected daily beginning five days before the start of the treatment, during the treatment and until 28 days after the last treatment. Ten eggs per day were randomly selected for sampling at each time point coming from the first ten hens having regularly laid one egg per day and throughout all study. Samples were a mixture of albumen and yolk of each egg selected at each time point. Only erythromycin A was measured in eggs with the LC/MS/MS method used in the above studies. The results are presented in Table 7.

Table 7: Mean concentrations of erythromycin A in eggs of laying hens treated with 20 mg/kg/day for seven days.

Sampling time	Mean concentration (µg/kg)
Beginning of treatment	
Day 1	< LOD
Day 2	< LOQ
Day 3	< LOQ
Day 4	72 ± 11.1
Day 5	$135 \pm nc$
Day 6	59 ± 7.1
Day 7	68 ± 18.3
End of treatment	
1 day post treatment	59 ± 0.7
2-8 days post treatment	< LOQ
9-21 days post treatment	< LOD

Note: < LOD: below the limit of detection (0.9 µg/kg);

< LOQ: below the limit of quantification (50 µg/kg);

nc: not calculated

Erythromycin could be quantified only 1 day after the end of the treatment. After this time, the concentrations of the residues were below the LOQ (50µg/kg). The study demonstrated that, whatever the duration of the administration, the administration of 20mg/kg b.w./day results in concentrations of erythromycin in eggs no higher that those found with fewer days of administration.

Residue Depletion Studies in Turkeys

- Treatment for three consecutive days at the maximum recommended dose (MP/5814/0225)

Thirty four white turkeys were treated by oral administration of 20 mg/kg/day of erythromycin thiocyanate 20% oral powder via drinking water for 3 consecutive days. All animals belonged to the same strain and were healthy when they received the treatment. Animals received a daily ration of commercial concentrated feed and had free access to water. They weighed 2289 ± 309 g at the beginning of the treatment. Turkeys were selected for the acclimatization period (14 days) before the beginning of the treatment. Six animals per sampling time were collected at 3, 4, 5, 6 and 8 days after treatment (1, 2, 3, 4 and 6 days after the last dose), 4 birds used as controls were sampled at six days after the end of the treatment. Individual edible tissues collected from animals were: whole liver, both kidneys, 400g of pectoral muscle, and approximately 20-50g of fat+skin in natural proportions. The LC/MS/MS

assay method for the detection of residues in turkeys was adapted from the chicken method. Three days after the end of the treatment, residues were detectable only in liver (166±64 µg/kg). At this time, residues were measured only in one sample of kidney, muscle and fat. After four days post-treatment, all concentrations of residues were below the LOQ or the LOD in all tissues. The results are presented in Table 8.

Table 8: Mean concentrations of erythromycin A in edible tissues of turkeys treated with 20mg/kg/day for three days.

Sampling time after treatment	Muscle (µg/kg)	Fat/skin (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)
3 days	266*	318*	166 ± 63.6	424*
4, 5,6 and 8 days	< LOQ	< LOQ	< LOQ	< LOD

Note: * only one value;< LOQ: below the quantification limit (100 µg/kg for all tissues); < LOD: below the detection limit (4 µg/kg for skin/fat and 3 µg/kg for all other tissues).

METHODS OF ANALYSIS

Erythromycin A, B and C and N-methyl-erythromycin A by a LC/MS/MS method

Chicken (MPK/5814 /9812 and validation in MPK/erythromycin/9957).

Extraction procedure for muscle, liver and kidney: 1 g thawed tissue was shaken with 1 ml of double distilled water using a vortex mixer. Nine ml of acetonitrile was added and the mixture vortexed for an additional 20 seconds. The mixture was shaken for 10 minutes using a mechanical shaker. After decantation, 100µl of the supernatant was added to 300µl of the dilution solution and 15µl was injected into the chromatographic system.

Extraction procedure for skin/fat: 1 g frozen tissue was shaken with 20 ml of acetonitrile for 20 seconds using a vortex mixer followed by 10 minutes using a mechanical linear shaker. After decantation, 1 ml of supernatant and 1 ml of double distilled water and 2 ml of n-hexane were added. The mixture was shaken 10 minutes using a mechanical shaker, centrifuged at 2500 rpm for 5 minutes, n-hexane was decanted off and 15 µl of solution were injected onto the chromatographic system.

Chromatographic procedure: 15 µl was injected onto the column using the autosampler. The column used was an RP 18ec 30 x 4 mm, 5 µm. The elution was done under isocratic conditions with acetonitrile/double distilled water 50:50 v/v with 1% formic acid. The retention times were 2.7 min for erythromycin A, 3.1 min for erythromycin B, 2.0 min for erythromycin C and 2.4 min for N-desmethyl-erythromycin A. Detection was done by mass-mass using a quadripole instrument with an electrospray source in the positive ionization mode. Multiple Reaction Monitoring (MRM) was applied and the following transition ions were monitored 734.6 →576.6 for erythromycin A, and 718.6 →565.2 for erythromycin B, 720.6 →576.5 for erythromycin C, and 720.5 →562.2 for N-desmethyl-erythromycin A. No internal standard was used. Quantification of both products was done after interpolation of unknown sample peak areas against the theoretical concentrations of calibration curves. Results were expressed as free erythromycin A and N-desmethyl-erythromycin A (µg/kg). The method was validated using the following criteria consistent with internationally recognized guidelines.

Specificity: The method was found specific from endogenous compounds, erythromycin B and C, N-desmethyl-erythromycin A, tylosin, tilmicosin and spiramycin.

Linearity: The linearity of detector response was assessed in the four tissues matched with standard solutions using 100-5000 µg/kg. The correlation coefficients were 0.9996, 0.9993, 0.9998 and 0.9993

for erythromycin A in muscle, liver, kidney and skin/fat respectively and 0.9979, 0.9987, 0.9992 and 0.9976 for N-methyl-erythromycin A in the same tissues, respectively.

Stability: Stability was demonstrated at a concentration of 2000 µg/kg after 3 freeze-thaw cycles.

The within-day precision, day-to-day precision, accuracy, recovery rate, LOQ and LOD are presented in the following tables.

Table 9: Validation parameters for erythromycin A in chicken tissues

Erythromycin A	Muscle	Kidney	Fat / skin	Liver
Concentration (µg/kg)	100 - 5000	100 - 5000	100 - 5000	100 - 5000
Within-day precision (%)	1.95 - 7.57	2.30 - 7.24	2.51 - 7.80	2.89 - 8.19
Day-to-day precision (%)	2.28 - 7.57	2.30 - 7.24	2.51 - 9.36	4.31 - 13.6
Accuracy (E%)	-1.5 ≤ E% ≤ +2.0	-2.0 ≤ E% ≤ +2.4	-7.0 ≤ E% ≤ +10.1	-3.3 ≤ E% ≤ +3.0
Recovery rate (%)	> 98.9	> 92.1	> 87.4	> 98.1
LOQ (µg/kg)	100	100	100	100
LOD (µg/kg)	3	25	5	30

Table 10: Validation parameters for N-desmethyl-Erythromycin A in chicken tissues

N-desmethyl-Erythromycin A	Muscle	Kidney	Fat / skin	Liver
Range (µg/kg)	100 - 5000	100 - 5000	100 - 5000	100 - 5000
Within-day precision (%)	2.56 - 6.37	1.95 - 7.19	2.61 - 9.33	2.48 - 6.85
Day-to-day precision (%)	2.62 - 7.15	1.95 - 7.19	2.61 - 9.33	3.86 - 9.47
Accuracy (E%)	-3.0 ≤ E% ≤ +3.4	-1.2 ≤ E% ≤ +3.8	-4.0 ≤ E% ≤ +10.4	-4.2 ≤ E% ≤ +4.0
Recovery rate (%)	> 99.1	> 96.8	> 81.7	> 97.3
LOQ (µg/kg)	100	100	100	100
LOD (µg/kg)	3	25	24	48

Turkey (MPK/5814/0225)

The analytical method was adapted from the chicken method described above. It was validated using the criteria noted above. The method was partially validated as noted below. The retention time was approximately 3.6 min for erythromycin A.

Specificity: The method was found specific from endogenous compounds, erythromycin B and C, N-desmethyl erythromycin A, tylosin, tilmicosin and spiramycin.

Linearity: The linearity of detector response was assessed in the four tissues matched with standard solutions in the range of 100-5000 µg/kg. The correlation coefficients were 0.9998, 0.9997, 0.9988 and 0.9993 for erythromycin A in muscle, liver, kidney and skin/fat. The within-day precision, accuracy, recovery rate, LOQ and LOD are presented in Table 11.

Table 11: Validation parameters for erythromycin A in turkey tissues

Erythromycin A	Muscle	Liver	Kidney	Fat / skin
Range ($\mu\text{g}/\text{kg}$)	100 - 5000	100 - 5000	100 - 5000	100 - 5000
Within-day precision (%)	1.1 - 2.3	1.6 - 4.6	1.1 - 2.2	4.9 - 11.2
Accuracy (E%)	-5.0 - 6.5	-8.0 - 8.4	-6.8 - 0.2	-3.3 - 8.8
LOQ ($\mu\text{g}/\text{kg}$)	100	100	100	100
LOD ($\mu\text{g}/\text{kg}$)	3	3	3	4

Eggs (MPK/ 5814/9908 and validation in MPK/erythromycin/9961)

Extraction procedure: 1g frozen mixed egg was shaken with 1 ml of double distilled water for 20 seconds using a vortex mixer. Nine ml of acetonitrile were added and the mixture was shaken for 20 seconds using a vortex mixer and for 10 minutes using a mechanical linear shaker. After decantation, 500 μl of supernatant were added to 500 μl of acetonitrile, 1 ml of double distilled water and 2 ml of n-hexane. The mixture was shaken 10 minutes using a mechanical shaker, centrifuged, the n-hexane was decanted off and 10 μl of solution were injected onto the chromatographic system.

Chromatographic procedure: 15 μl were injected onto a RP 18ec 30 x 4 mm, 5 μm column. The elution was obtained under isocratic conditions with acetonitrile/double distilled water 50:50 v/v with 1% formic acid. The retention times were 3 min for erythromycin A and 2.5 min for N- desmethyl-erythromycin A. Detection was done by mass-mass using a quadripole instrument with a turbo ionspray source in the positive ionization mode. MRM was applied and the following transition ions were monitored 734.6 \rightarrow 158.0 for erythromycin A and 720.5 \rightarrow 144.2 for N-desmethyl-erythromycin A. No internal standard was used. Quantification of both products was done after interpolation of unknown sample peak areas against the theoretical concentrations of calibration curves. Results were expressed as free erythromycin A and N-desmethyl-erythromycin A.

Specificity: The method was found specific from endogenous compounds, erythromycin B and C, N-desmethyl erythromycin A, tylosin, tilmicosin and spiramycin.

Linearity: The linearity of detector response was assessed matched with standard solutions in the range of 50-5000 $\mu\text{g}/\text{kg}$. The correlation coefficients were 0.9993 and 0.9997 for erythromycin A and N-methyl-erythromycin A respectively.

Stability: The stability of erythromycin A and N-desmethyl-erythromycin A was evaluated at the concentration level of 2000 $\mu\text{g}/\text{kg}$ after 3 freeze-thaw cycles. The mean concentration was approximately 104% of the mean reference value. Long term stability was evaluated in both compounds in a mixed frozen egg stored at -80 $^{\circ}\text{C}$ for 1 month at the concentration level of 2000 $\mu\text{g}/\text{kg}$. The mean concentration of the pool was 102% and 104% of the nominal value of erythromycin A and N-methyl-erythromycin A, respectively.

The within-day precision, day-to-day precision, accuracy, recovery rate, LOQ and LOD are presented in Table 12.

Table 12: Validation parameters for erythromycin A and N-desmethyl-erythromycin A in eggs

	Erythromycin A	N-desmethyl-Erythromycin A
Range (µg/kg)	50 - 5000	50 - 5000
Within-day precision (%)	1.76 - 9.87	1.10 - 7.79
Day-to-day precision (%)	1.76 - 11.87	1.11 - 7.79
Accuracy (E%)	-5.0 ≤ E% ≤ +7.8	-1.3 ≤ E% ≤ +2.0
Recovery rate (%)	> 82.6	> 71.9
LOQ (µg/kg)	50	50
LOD (µg/kg)	0.92	2.2

Microbiological assay method for erythromycin in chicken tissues and eggs

A microbiological method was used for the assay of erythromycin A and its related metabolites with potential microbiological activity in edible tissues (muscle, kidney, liver and fat/skin) of chicken (MPK/erythromycin/9957) and eggs (MPK/Erythromycin/9961).

Extraction procedure: 1 g of test sample was vortexed with 4 ml of acetonitrile, placed in an ultrasonic bath for five minutes and on a liner shaker for ten minutes. The extract was centrifuged at 7500g for 10 minutes and the supernatant transferred into a disposable tube. Two wells of each Petri dish were filled with this mixture.

Petri dishes: Plates were prepared (diameter 100mm) using agar medium and *Micrococcus Luteus ATCC9341* as test organism. For each point, two plates with two replicates per plates were used. The diameter of bacterial growth inhibition was the measured response. Amounts of erythromycin were calculated by interpolating the inhibition diameter into the standard curve. The method has been validated according to the following criteria.

Specificity: The method shows specificity between erythromycin and endogenous component of the tissues. Three different matrixes are tested under experimental conditions for each tissue. No inhibition zone was observed for any tissue.

Linearity in chicken tissues: The linearity of detector response was assessed in the four tissues matched with standard solutions in the range from 100 to 2000µg/kg. The correlation coefficients were 0.9994, 0.9513, 0.9571 and 0.9969 for erythromycin A in muscle, liver, kidney and skin/fat, respectively. Other validation parameters are presented in the following tables for each tissue.

Linearity in eggs: The linearity of detector response was assessed matched with standard solutions in the range from 100 to 2000µg/kg. The correlation coefficient was 0.9567 as average of 9 standard curves. Other validation parameters are presented in the following tables.

Table 13: Validation parameters of the microbiological method for erythromycin in chicken Table 13-A

Validation parameters for kidney							
Concentrations (µg/kg).	100	200	500	1000	1500	2000	Overall
Recovery rate (%) n=9	85.5	86.5	88.2	85.1	84.8	86.1	86.0
Repeatability, CV _r % n=3 per day	5.9	9.4	7.8	10.8	5.7	6.0	-
Intermediate precision, CV _r %/ 3 days	12.9	12.8	13.5	10.8	5.7	10.5	-
Accuracy: difference (% value)	-5.11	3.78	7.07	4.02	-1.90	-4.27	0.60
Limit of Detection (LOD)				50 µg/kg			
Limit of Quantification (LOQ)				100 µg/kg			
- = not calculated							

Table 13-B

Validation parameters for fat + skin							
Concentrations ($\mu\text{g}/\text{kg}$).	100	200	500	1000	1500	2000	Overall
Recovery rate (%) n=9	101.5	102.9	100.0	97.5	97.1	96.6	99.2
Repeatability $\text{Cv}_r\%$ n=3 per day	3.7	5.6	4.6	7.5	6.9	6.7	-
Intermediate precision, $\text{Cv}_r\%$ / 3 days	3.7	5.6	5.4	7.5	6.9	6.7	-
Accuracy: difference (% value)	0.22	-0.22	-1.13	4.99	-3.44	0.67	0.18
Limit of Detection (LOD)	50 $\mu\text{g}/\text{kg}$						
Limit of Quantification (LOQ)	100 $\mu\text{g}/\text{kg}$						
- = not calculated							

Table 13-C

Validation parameters for muscle							
Concentrations ($\mu\text{g}/\text{kg}$).	100	200	500	1000	1500	2000	Overall
Recovery rate (%) n=9	94.1	94.0	93.6	92.6	93.1	95.9	93.9
Repeatability $\text{Cv}_r\%$ n=3 per day	9.4	13.3	7.9	4.6	7.3	4.5	-
Intermediate precision, $\text{Cv}_r\%$ / 3 days	9.8	13.3	7.9	4.6	7.3	6.2	-
Accuracy: difference (% value)	-3.22	5.22	2.53	-2.00	-1.45	1.06	0.36
Limit of Detection (LOD)	50 $\mu\text{g}/\text{kg}$						
Limit of Quantification (LOQ)	100 $\mu\text{g}/\text{kg}$						
- = not calculated							

Table 13-D

Validation parameters for liver							
Concentrations ($\mu\text{g}/\text{kg}$).	100	200	500	1000	1500	2000	Overall
Recovery rate (%) n=9	86.6	83.7	88.5	87.8	87.8	88.8	87.2
Repeatability, $\text{Cv}_r\%$ n=3 per day	5.2	7.6	7.0	8.8	6.1	6.3	-
Intermediate precision, $\text{Cv}_r\%$ / 3 days	9.2	7.9	13.3	8.8	6.2	6.3	-
Accuracy: difference (% value)	0.22	-5.72	9.47	4.04	1.26	-6.51	0.46
Limit of Detection (LOD)	50 $\mu\text{g}/\text{kg}$						
Limit of Quantification (LOQ)	100 $\mu\text{g}/\text{kg}$						
- = not calculated							

Table 14: Validation parameters of a microbiological assay method for erythromycin in eggs

Validation parameters							
Concentrations ($\mu\text{g}/\text{kg}$).	100	200	500	1000	1500	2000	Overall
Recovery rate (%) n=9	87.5	93.5	94.2	93.4	92.4	92.8	92.3
Repeatability, $\text{Cv}_r\%$ n=3 per day	2.9	2.6	4.7	3.9	4.3	4.6	-
Intermediate precision, $\text{Cv}_r\%$ / 3 days	5.0	4.5	5.1	5.7	4.5	4.6	-
Accuracy : difference (% value)	-4.89	3.11	3.87	5.77	-2.21	-4.59	0.18
Limit of Detection (LOD) :	50 $\mu\text{g}/\text{kg}$						
Limit of Quantification (LOQ) :	100 $\mu\text{g}/\text{kg}$						
- = not calculated							

Wang, et al. (2005) published a method for determination of five macrolide antibiotic residues in eggs (spiramycin, tilmicosin, oleandomycin, erythromycin and tylosin) using liquid chromatography/electrospray ionization tandem mass spectrometry. Data acquisition under MS/MS was achieved by multiple reaction monitoring of two or three fragment ion transitions for both quantification and confirmation. A full experimental design was used to study the measurement uncertainty arising from intermediate precision and trueness or proportional bias. The overall recoveries of spiramycin, tilmicosin, oleandomycin, erythromycin and tylosin at fortified levels of 60, 100, 200 and 300 µg/kg were 96.8, 98.2, 98.3, 98.8 and 95.4, respectively. The method detection limits (S/N ≥ 3:1) of five macrolides were <1.0 µg/kg).

APPRAISAL

Erythromycin is an old drug. It was first reviewed by the Committee in 1968. No ADI was established but acceptable levels of residues were defined in milk (0-0.04 mg/ml) and meat (0-0.3 mg/kg). Erythromycin is a mixture of three compounds produced during fermentation. The main product is erythromycin A with small portions of B (≤ 5%) and C (≤ 5%). In veterinary medicine, erythromycin is used for the treatment of clinical and subclinical mastitis in lactating cows, for the treatment of infectious diseases due to erythromycin sensitive bacteria (cattle, sheep, swine, and poultry) and for the treatment of chronic diseases due to mycoplasma in poultry. The maximum recommended therapeutic dose in veterinary use is 20/mg/kg/day as erythromycin base.

Data from pharmacokinetic and metabolic studies in experimental and target animals and humans were submitted for evaluation by the Committee together with two earlier residue studies in calves and chickens. Three new non radiolabeled residue depletion studies in chickens, laying hens and turkeys treated with erythromycin and the description and validation of the analytical procedures employed were provided.

Erythromycin is rather slowly absorbed in humans, rats, cattle and chicken with some differences related to the mode of administration (IM, IV and oral), the salt form and the coating of the administered compound. Protein binding is variable ranging from 90% in man to 38-45% in cattle. The major site of absorption is rats, dogs and humans is the small intestine. Erythromycin is only slightly absorbed in the stomach. The tissue concentrations are higher than in serum and persist longer. Erythromycin is mainly excreted in the faeces through the bile, 37 to 43 % of the dose was recovered in the intestinal tract plus faeces of rats. Urinary excretion ranged from 10 to 36 % in different species (human, rats and dogs).

Erythromycin is rapidly metabolized in the liver, mainly through an N-demethylation process in a variety of species of rodents, ruminants and humans. N-desmethyl-erythromycin was the major metabolite and the only microbiologically active metabolite of erythromycin. However, its antimicrobial activity is low and the only form of erythromycin known to be active *in vivo* is erythromycin free base.

Two studies were performed in 1988 in calves and poultry using erythromycin thiocyanate. In poultry, the residues of erythromycin were determined in chicken tissues after administration by the oral route in drinking water for 3 consecutive days. In a similar study the residues of erythromycin in whole eggs were determined following medication administered to laying hens for 7 consecutive days in drinking water. The concentration of the erythromycin in chicken tissues declined to values below the limit of detection three days after the end of treatment and six days after the end of treatment in whole eggs.

There was no radiolabelled study reported, however, four new residue depletion studies with unlabelled erythromycin were performed in poultry. The route of administration, the dose and the species are those intended for therapeutic use.

The residues with microbiological activity were measured in chickens and laying hens by a microbiological plate assay using agar medium and *Micrococcus luteus* ATCC9341 as test organism

(LOD: 50 µg/kg for all chicken tissues and eggs; LOQ: 100 µg/kg for all chicken tissues and eggs). The concentration of erythromycin A and its metabolites were simultaneously assayed using a LC/MS/MS method. For chickens, erythromycin A, B and C, the LOD are as follows: 25 µg/kg for kidney, 30 µg/kg for liver, 3 µg/kg for muscle and 5 µg/kg for skin + fat and the LOQ is 100 µg/kg in all tissues. For N-desmethyl-erythromycin A, the LOD are as follows: 25 µg/kg for kidney, 48 µg/kg for liver, 5 µg/kg for muscle and 24 µg/kg for skin + fat and the LOQ is 100µg/kg in all tissues. For eggs, the erythromycin LOQ is 50 µg/kg and the LOD is 0.92 µg/kg. For turkey, the erythromycin LOQ is 100 µg/kg for all tissues and the LOD is 4 µg/kg for skin/fat and 3 µg/kg for all other tissues.

In the chicken studies, all tissue residue depletion results showed that from day 1 to day 3 after the end of the treatment period, low concentrations of erythromycin A and N-desmethyl-erythromycin A were measured in only a few liver samples. Prolonged treatment for up to eight days resulted in the same tissue residue concentration characteristics. In eggs, during the three days of treatment, mean concentrations of erythromycin A ranged from 109 µg/kg (day 1) to 83 µg/kg (day 3) measured by the LC/MS/MS method. Taking into account the standard deviation, there are no significant differences in residue concentration between days. The egg residues of erythromycin after the end of the treatment were 57±6 µg/kg, 71± 6 µg/kg and 54± nc µg/kg for days 1, 2 and 3, respectively.

Results show that erythromycin A and its metabolite N-desmethyl-erythromycin A were the major compounds observed (the ratio erythromycin/total active microbiological metabolites was 0.33 and the ratio of N-desmethyl-erythromycin/total active microbiological metabolites was 0.75). Erythromycin could only be quantified in 25% of eggs at day 1 and 12.5% of the eggs at day 2. Concentrations of erythromycin after the end of the treatment measured by LC/MS/MS were near the LOQ (50 µg/kg). N-desmethyl-erythromycin was present at higher concentrations but had very low antimicrobial activity. Both compounds were below the LOQ at 6 days after the end of treatment. Erythromycin A was identified as the marker residue for eggs.

Tissue residue depletion studies in turkeys yielded similar results. The LC/MS/MS assay method was adapted from the chicken method. At three days after the end of the treatment, residues were detected only in liver (166±64 µg/kg). At this time, residues were measured only in one sample of kidney, muscle and fat. Four days post-treatment, all concentrations of residues were below the LOQ or the LOD.

The 66th meeting of the Committee agreed to apply a new approach to estimate chronic (long term) exposure using the median residue concentration of residues, using the standard food basket, in addition to the historically used theoretical maximum daily intake calculation based on the MRL. For numerical values reported below the LOD and the LOQ, one-half the analytical limit was applied to each, respectively, for estimating daily exposure concentrations. For erythromycin, the summary of the median residue concentrations in chickens, turkeys and eggs are presented in the following tables, derived from using individual values in all studies using an Excel spreadsheet.

Table 15a: Median residues in chicken and turkey tissues (µg/kg)

Tissue & Time	Chicken			Turkey			
	12 hours	1 day	2 days	1 day	2 days	3 days	4 days
Muscle	1.5	1.5	1.5	50	50	1.5	1.5
Liver	15	15	15	50	50	15	15
Kidney	12.5	12.5	12.5	50	12.5	12.5	12.5
Fat+Skin	50	2.5	2.5	50	25.8	2.5	2.5

Table 15b: Median residues in chicken eggs (µg/kg)

Time (days)											
0 day	1 day	2 days	3 days	4 days	5 days	6 days	7 days	8 days	9 days	10 days	11 days
0.45	25	25	25	25	25	25	41	25	25	25	25

The LC/MS/MS analytical methods provided are applicable for the determination of residues of erythromycin in chicken and turkey tissues (muscle, fat, kidney and liver) and eggs. The method was validated.

MAXIMUM RESIDUE LIMITS

In recommending MRLs for erythromycin, the Committee considered the following factors:

- The marker residue is erythromycin A. Metabolites exhibited little or no microbiological activity.
- Only MRLs in poultry tissues and eggs were considered.
- Residue studies in bovine and ovine tissues suggest mean ratios of marker residue to total residues for tissues to be 0.7 for muscle and kidney, 0.5 for liver and 0.85 for fat. The ratio was not always available for all species and when it was, it was only available based on few individual data. From submitted studies, the marker residue to total residues for eggs was estimated to be 0.33.
- Residue depletion studies generated very limited numbers of residue concentrations above the limit of quantification for all studies in chickens, turkeys and eggs.
- A validated LC/MS/MS method is available with a limit of quantification of 100 µg/kg for all tissues and 50 µg/kg for eggs. The limit of quantification of the microbiological method is 100 µg/kg for all tissues and eggs.
- For residue concentrations reported below the LOD and the LOQ, one-half the analytical limit was applied to each, respectively, for estimating daily exposure concentrations.
- The ADI for erythromycin A was 0 – 0.7 µg/kg body weight, equivalent to 42 µg per person per day.

Noting the factors noted above, the Committee recommended MRLs of 100 µg/kg for chicken and turkey muscle, liver, kidney and fat/skin and 50µg/kg for eggs at the limit of quantification of the LC/MS/MS method, measured as erythromycin A.

Applying the MRLs and the standard food basket, the theoretical maximum daily intake is 55 µg, equivalent to approximately 130% of the ADI. The 66th meeting of the Committee agreed to apply the principle of using median residue concentrations to better estimate long-term (chronic) exposures to residues. Estimated daily intake (EDI) values were determined using median residue values for each tissue from each food-producing species for which data were available. Median residue values were determined using an Excel[®] spreadsheet program. Where residue values were below the LOD or LOQ of the validated method, values of ½ the LOD and ½ the LOQ, respectively, were used in the calculations. Applying the highest estimated median residue concentrations in turkeys (50 µg/kg in all tissues) – the median residue concentrations in turkey tissues were higher than the corresponding median residue concentrations in chicken tissues - and eggs (41µg/kg), the estimated daily intake is 29.1 µg/kg, equivalent to approximately 69% of the ADI. Results are shown in Table 16.

Table 16: Estimated daily intake

Tissue	Median intake ($\mu\text{g}/\text{kg}$)	Standard Food Basket (kg)	Daily intake (μg)
Muscle	50	0.3	15
Liver	50	0.1	5
Kidney	50	0.05	2.5
Fat	50	0.05	2.5
Eggs	41	0.1	4.1
Total			29.1

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